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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

**Achim H. Krotz and Vasulinga T.
Ravikumar**

Serial No.: Not Yet Assigned

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For: IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES

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Box Patent Application
 Provisional Design Sequence

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

A Utility Patent Application.

If this is a continuing application, please check appropriate box:

continuation divisional continuation-in-part of prior application number
_____/_____.

A Provisional Patent Application.

A Design Patent Application (submitted in duplicate).

Including the following:

Provisional Application Cover Sheet.

New or Revised Specification, including pages 1 to 36 containing:

Specification

Claims

Abstract

A copy of earlier application Serial No. _____ Filed _____ to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

Please enter the following amendment to the Specification under the Cross Reference to Related Applications section (or create such a section) : "This Application is a continuation or divisional of Application Serial No. _____ filed _____

_____. "

Signed Statement attached deleting inventor(s) named in the prior application.

A Preliminary Amendment.

_____ Sheets of Formal Informal Drawings.

Petition to Accept Photographic Drawings.

Petition Fee

An Executed Unexecuted Declaration or Oath and Power of Attorney.

An Associate Power of Attorney.

An Executed Copy of Executed Assignment of the Invention to _____

A Recordation Form Cover Sheet.

Recordation Fee - \$40.00.

Priority is claimed under 35 U.S.C. § 119 of application Serial No. _____ filed _____ in _____ (country).

A Certified Copy of each of the following applications for which priority is claimed:

is enclosed.

has been filed in prior application Serial No. _____ filed _____.

An Executed Unexecuted Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27

is enclosed.

has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

Diskette Containing DNA/Amino Acid Sequence Information.

Statement to Support Submission of DNA/Amino Acid Sequence Information.

Letter of Reference to Computer Readable Form.

Information Disclosure Statement.

Attached Form 1449.

Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

A copy of Petition for Extension of Time as filed in the prior case.

Appended Material as follows: _____

Return Receipt Postcard (should be specifically itemized).

Other as follows: _____

FEE CALCULATION

		SMALL ENTITY		NOT SMALL ENTITY	
		RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION		\$75.00	\$	\$150.00	\$
DESIGN APPLICATION		\$165.00	\$	\$330.00	\$
UTILITY APPLICATIONS BASE FEE		\$395.00	\$395	\$790.00	\$
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS					
	No. Filed	No. Extra			
TOTAL CLAIMS	41 - 20 =	21	\$11 each	\$231	\$22 each
INDEP. CLAIMS	2 - 3 =	0	\$41 each	\$0	\$82 each
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			\$135	\$0	\$270
ADDITIONAL FILING FEE				\$231	\$
TOTAL FILING FEE DUE				\$626	\$

Two (2) Checks are enclosed in the amounts of \$ 604 & \$22 totaling \$626.

The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

- The foregoing amount due.
- Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.
- Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).
- The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: February 26, 1998



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IMPROVED METHODS FOR SYNTHESIS OF OLIGONUCLEOTIDES

FIELD OF THE INVENTION

The present invention is directed to improved methods for synthesis of oligonucleotides and other 5 phosphorus-linked oligomers, using aromatic solvents, alkyl aromatic solvents, halogenated aromatic solvents, halogenated alkyl aromatic solvents, or aromatic ether solvents. Oligomers synthesized using the methods of the invention are useful for diagnostic reagents, research 10 reagents and in therapeutics.

BACKGROUND OF THE INVENTION

It is well known proteins are significantly involved in many of the bodily states in multicellular organisms, including most disease states. Such proteins, 15 either acting directly or through their enzymatic or other functions, contribute in major proportion to many diseases and regulatory functions in animals and man. For disease states, classical therapeutics has generally focused upon interactions with such proteins in efforts to moderate their 20 disease-causing or disease-potentiating functions. In newer therapeutic approaches, modulation of the production of such proteins is desired. By interfering with the production of proteins, the maximum therapeutic effect might be obtained with minimal side effects. It is the general object of such 25 therapeutic approaches to interfere with or otherwise

modulate gene expression which would lead to undesired protein formation.

One method for inhibiting specific gene expression is with the use of oligonucleotides, especially 5 oligonucleotides which are complementary to a specific target messenger RNA (mRNA) sequence.

Transcription factors interact with double-stranded DNA during regulation of transcription. Oligonucleotides can serve as competitive inhibitors of transcription factors 10 to modulate the action of transcription factors. Several recent reports describe such interactions (see Bielinska, A., et. al., *Science*, 1990, 250, 997-1000; and Wu, H., et. al., *Gene*, 1990, 89, 203-209).

In addition to functioning as both indirect and 15 direct regulators of proteins, oligonucleotides have also found use in diagnostic tests. Such diagnostic tests can be performed using biological fluids, tissues, intact cells or isolated cellular components. As with gene expression inhibition, diagnostic applications utilize the ability of 20 oligonucleotides to hybridize with a complementary strand of nucleic acid. Hybridization is the sequence specific hydrogen bonding of oligonucleotides, via Watson-Crick and/or Hoogsteen base pairs, to RNA or DNA. The bases of such base pairs are said to be complementary to one another.

25 Oligonucleotides are also widely used as research reagents. They are useful for understanding the function of many other biological molecules as well as in the preparation of other biological molecules. For example, the use of oligonucleotides as primers in polymerase chain 30 reactions (PCR) has given rise to an expanding commercial industry. PCR has become a mainstay of commercial and research laboratories, and applications of PCR have multiplied. For example, PCR technology is used in the fields of forensics, paleontology, evolutionary studies and 35 genetic counseling. Commercialization has led to the development of kits which assist non-molecular biology-trained personnel in applying PCR. Oligonucleotides, both

natural and synthetic, are employed as primers in PCR technology.

Laboratory uses of oligonucleotides are described generally in laboratory manuals such as *Molecular Cloning, A Laboratory Manual*, Second Ed., J. Sambrook, et al., Eds., Cold Spring Harbor Laboratory Press, 1989; and *Current Protocols In Molecular Biology*, F. M. Ausubel, et al., Eds., Current Publications, 1993. Such uses include Synthetic Oligonucleotide Probes, Screening Expression Libraries with Antibodies and Oligonucleotides, DNA Sequencing, In Vitro Amplification of DNA by the Polymerase Chain Reaction and Site-directed Mutagenesis of Cloned DNA (see Book 2 of *Molecular Cloning, A Laboratory Manual*, *ibid.*) and DNA-Protein Interactions and The Polymerase Chain Reaction (see Vol. 2 of *Current Protocols In Molecular Biology*, *ibid.*).

Oligonucleotides can be custom-synthesized for a desired use. Thus a number of chemical modifications have been introduced into oligonucleotides to increase their usefulness in diagnostics, as research reagents and as therapeutic entities. Such modifications include those designed to increase binding to a target strand (i.e. increase their melting temperatures, (T_m)); to assist in identification of the oligonucleotide or an oligonucleotide-target complex; to increase cell penetration; to stabilize against nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotides; to provide a mode of disruption (terminating event) once sequence-specifically bound to a target; and to improve the pharmacokinetic properties of the oligonucleotides.

Thus, it is of increasing value to prepare oligonucleotides and other phosphorus-linked oligomers for use in basic research or for diagnostic or therapeutic applications. Consequently, and in view of the considerable expense and time required for synthesis of specific oligonucleotides, there has been a longstanding effort to develop successful methodologies for the preparation of

specific oligonucleotides with increased efficiency and product purity.

Synthesis of oligonucleotides can be accomplished using both solution phase and solid phase methods.

5 Oligonucleotide synthesis via solution phase in turn can be
accomplished with several coupling mechanisms. However,
solution phase chemistry requires purification after each
internucleotide coupling, which is labor intensive and time
consuming.

10 The current method of choice for the preparation of
naturally occurring oligonucleotides, as well as modified
oligonucleotides such as phosphorothioate and phosphoro-
dithioate oligonucleotides, is via solid-phase synthesis
wherein an oligonucleotide is prepared on a polymer support
15 (a solid support) such as controlled pore glass (CPG);
oxalyl-controlled pore glass (see, e.g., Alul, et al.,
Nucleic Acids Research 1991, 19, 1527); TENTAGEL Support,
(see, e.g., Wright, et al., *Tetrahedron Letters* 1993, 34,
3373); or POROS, a polystyrene resin available from
20 Perceptive Biosystems.

Solid-phase synthesis relies on sequential addition of nucleotides to one end of a growing oligonucleotide chain. Typically, a first nucleoside (having protecting groups on any exocyclic amine functionalities present) is attached to an appropriate glass bead support and activated phosphite compounds (typically nucleotide phosphoramidites, also bearing appropriate protecting groups) are added stepwise to elongate the growing oligonucleotide. The nucleotide phosphoramidites are reacted with the growing oligonucleotide using "fluidized bed" technology to mix the reagents. The known silica supports suitable for anchoring the oligonucleotide are very fragile and thus cannot be exposed to aggressive mixing. Brill, W. K. D., et al. *J. Am. Chem. Soc.*, 1989, 111, 2321, disclosed a procedure wherein an aryl mercaptan is substituted for the nucleotide phosphoramidite to prepare phosphorodithioate oligonucleotides on glass supports.

Additional methods for solid-phase synthesis may be found in Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents Nos. 4,725,677 and Re. 34,069.

5 While current solid-phase syntheses are suitable for preparing relatively small quantities of oligonucleotides, *i.e.*, from about the micromolar (μmol) to millimolar (mmol) range, they typically are not amenable to the preparation of the larger quantities of oligonucleotides 10 necessary for biophysical studies, pre-clinical and clinical trials and commercial production. Currently, to synthesize more than about three fourths of a mmol of oligonucleotide it is necessary to do sequential syntheses. A general review of solid-phase versus solution-phase oligonucleotide 15 synthesis is given in the background section of Urdea, *et al.* U. S. Patent No. 4,517,338, entitled "Multiple Reactor System And Method For Oligonucleotide Synthesis".

Large-scale preparation of oligonucleotides can be carried out by solution-phase techniques. One such solution 20 phase preparation utilizes phosphorus triesters. Yau, E.K., *et al.*, *Tetrahedron Letters*, 1990, 31, 1953, report the use of phosphorous triesters to prepare thymidine dinucleoside and thymidine dinucleotide phosphorodithioates. The phosphorylated thymidine nucleoside intermediates utilized 25 in the synthesis were obtained by treatment of commercially available 5'-O- dimethoxytritylthymidine-3'- [(β -cyanoethyl)-N,N-diisopropyl]-phosphoramidite first with either 4-chloro or 2,4-dichlorobenzylmercaptan and tetrazole, and then a saturated sulfur solution. The resulting phosphorodithioate 30 nucleotide was then reacted via the triester synthesis method with a further thymidine nucleoside having a free 5'-hydroxyl.

Brill, W.K.D., *et al.* ., *J. Am. Chem. Soc.*, 1991, 113, 3972, disclose that treatment of a phosphoramidite such 35 as N,N-diisopropyl phosphoramidite with a mercaptan such as 4-chloro or 2,4-dichlorobenzylmercaptan in the presence of tetrazole yields a derivative suitable for preparation of a

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phosphoro-dithioate as a major product and a derivative suitable for preparation of a phosphorithioate as a minor product.

Further details of methods useful for preparing 5 oligonucleotides may be found in Sekine, M., etc al., *J. Org. Chem.*, 1979, 44, 2325; Dahl, O., *Sulfur Reports*, 1991, 11, 167-192; Kresse, J., et al. ., *Nucleic Acids Research*, 1975, 2, 1-9; Eckstein, F., *Ann. Rev. Biochem.*, 1985, 54, 367-402; and Yau, E.K. U.S. Patent No. 5,210,264.

10 Methods for synthesizing oligonucleotides using intermediates having phosphorus-containing covalent linkages involve the protection of the 5'-hydroxyl group of a nucleoside by forming trityl or substituted trityl or triarylaklyl derivatives. The protecting groups are later 15 removed under acidic conditions to yield the free 5'-hydroxyl group. The hydroxyl group can then be further reacted to give a coupled product.

The removal of trityl and other protecting groups is generally carried out in the presence of halogenated 20 solvents such as dichloromethane or dichloroethane. However, the use of such halogenated solvents is undesirable for several reasons, particularly in relatively large scale applications such as the manufacture of oligonucleotides or 25 analogs use as antisense agents. Consequently, there remains a need for methods of synthesis of oligonucleotides which provide improved efficiency and reduced disposal problems. The present invention is directed to these, as well as other, important ends.

SUMMARY OF THE INVENTION

30 The present invention provides improved methods for the preparation of oligonucleotides.

In some preferred embodiments the present invention provides methods for the preparation of a phosphorus-linked oligomer comprising the steps of:

35 (a) providing a solid support;
(b) attaching a 5'-O-protected nucleoside to the

solid support;

(c) deprotecting the 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the 5 solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;

(d) reacting the deprotected 5'-hydroxyl with an 5'-protected activated phosphorus compound to produce a 10 covalent linkage therebetween;

(e) oxidizing or sulfurizing the covalent linkage to form a phosphodiester, phosphorothioate, phosphorodithioate or H-phosphonate linkage;

(f) repeating steps c through e at least once for 15 subsequent couplings of additional activated phosphorus compounds, to produce the completed phosphorus-linked oligomer; and

(g) cleaving the oligomer from the solid support.

Some preferred embodiments of the methods of the 20 invention further comprise the step of capping remaining reactive sites with a solution containing a capping reagent.

In some preferred embodiments, the oxidation or sulfurization step is performed after each iteration of steps (c) and (d). In other preferred embodiments, a single 25 oxidation or sulfurization step is performed after the final iteration of steps (c) and (d).

In some preferred embodiments of the methods of the invention, solvent in step (c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether. In more 30 preferred embodiments the solvent in step (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether, with benzene, toluene or *o*-, *m*- or *p*-xylene being more preferred, and toluene being particularly preferred.

35 In other preferred embodiments of the methods of the invention, the solvent in step (c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent,

with chlorobenzene or benzotirfluoride being especially preferred.

In some preferred embodiments of the methods of the invention the activated phosphorus compound is an activated 5 mononucleotide, an activated dinucleotide, or an activated polynucleotide.

In further preferred embodiments of the method of the invention, the activated phosphorus compound is a 5'-protected nucleoside phosphoramidite or a 5'-protected 10 activated H-phosphonate nucleoside.

In some preferred embodiments of the methods of the invention, the 5'-protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphorus compound is independently trityl, monomethoxy trityl, 15 dimethoxytrityl, trimethoxytrityl, 2-chlorotriyl, DATE, TBTr, Pixyl or Moxyl, with trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl MOX) being more preferred, and dimethoxy trityl being especially preferred.

20 In some preferred embodiments of the methods of the invention, the phosphorus-linked oligomer is a phosphodiester, phosphorothioate phosphorodithioate, or H-phosphonate oligonucleotide.

In some preferred embodiments of the methods of the 25 invention, the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid.

30 In further preferred embodiments the solvent in step (c) further comprises an additive, which is preferably an alcohol, with from 0% to about 30% methanol, ethanol, 2-propanol, t-butyl alcohol, t-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof being especially preferred.

35 In some further preferred embodiments, the 5'-protected activated phosphorus compound is a 5'-protected activated H-phosphonate compound; and the phosphorus-linked

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oligomer is a H-phosphonate oligonucleotide.

In some preferred embodiments, the present invention provides methods for the preparation of a phosphorus-linked oligomer comprising the steps of:

- 5 a) providing a solid support;
- b) attaching a 5'-O-protected nucleoside to the solid support;
- c) contacting the protected 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic 10 acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;
- 15 d) reacting the deprotected 5'-hydroxyl with a 5'-protected activated phosphite compound to produce a phosphite linkage;
- e) oxidizing or sulfurizing the phosphite linkage to form a phosphodiester, phosphorothioate, or 20 phosphorodithioate linkage;
- f) repeating steps c through e at least once for subsequent couplings of additional activated phosphite compounds, to produce the completed phosphorus-linked oligomer; and
- 25 g) cleaving the oligomer from the solid support.

Some preferred embodiments of the methods of the invention further comprise the step of capping remaining reactive sites with a solution containing a capping reagent.

In some preferred embodiments of the methods of the 30 invention, solvent in step (c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether. In more preferred embodiments the solvent in step (c) is benzene, toluene, benzonitrile, o-, m- or p-xylene, mesitylene, or diphenyl ether, with benzene, toluene or o-, m- or p-xylene 35 being more preferred, and toluene being particularly preferred.

In other preferred embodiments of the methods of

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the invention, the solvent in step (c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent, with chlorobenzene or benzotriuoride being especially preferred.

5 In some preferred embodiments of the methods of the invention the activated phosphorus compound is a mononucleotide phosphoramidite, a dinucleotide phosphoramidite, or a polynucleotide phosphoramidite.

In further preferred embodiments of the method of the invention, the activated phosphorus compound is a 5'-protected nucleoside phosphoramidite or a 5'-protected activated H-phosphonate nucleoside.

In some preferred embodiments of the methods of the invention, the 5'-protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphorus compound is independently trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotriptyl, DATE, TBTr, Pixyl or Moxyl, with trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl MOX) being more preferred, and dimethoxy trityl being especially preferred.

In some preferred embodiments of the methods of the invention, the phosphorus-linked oligomer is a phosphodiester, phosphorothioate or phosphorodithioate oligonucleotide.

In some preferred embodiments of the methods of the invention, the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, 30 toluenesulfonic acid, or phenylphosphoric acid.

In further preferred embodiments the solvent in step (c) further comprises an additive, which is preferably an alcohol, with from 0% to about 30% methanol, ethanol, 2-propanol, *t*-butyl alcohol, *t*-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof being especially preferred.

In some particularly preferred embodiments, the

solvent in step (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether, with toluene being especially preferred; the activated phosphite compound is a mononucleotide phosphoramidite, a dinucleotide 5 phosphoramidite, or a polynucleotide phosphoramidite; the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl; the phosphorus linked oligomer is a phosphodiester, phosphorothioate or a phosphorodithioate 10 oligonucleotide; and the protic acid is dichloroacetic acid.

DETAILED DESCRIPTION

The present invention provides novel methods for the preparation of phosphorus-linked oligomers comprising the steps of:

- 15 (a) providing a solid support;
- (b) attaching a 5'-O-protected nucleoside to the solid support;
- (c) deprotecting the 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;
- 20 (d) reacting the deprotected 5'-hydroxyl with an 5'-protected activated phosphorus compound to produce a covalent linkage therebetween;
- (e) oxidizing or sulfurizing the covalent linkage to form a phosphodiester, phosphorothioate, phosphorodithioate or H-phosphonate linkage;
- 30 (f) repeating steps c through e at least once for subsequent couplings of additional activated phosphorus compounds, to produce the completed phosphorus-linked oligomer; and
- (g) cleaving the oligomer from the solid support.

35 It is generally preferable to perform a capping step after reaction of the deprotected 5'-hydroxyl with an

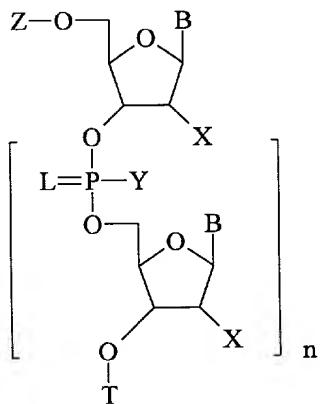
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5'-protected activated phosphorus compound. The capping step can be performed either before or after the oxidation or sulfurization step, and is generally known to provide benefits in the prevention of shortened oligomer chains, by 5 blocking chains that have not reacted in the coupling cycle. One representative reagent used for capping is acetic anhydride. Other suitable capping reagents and methodologies can be found in United States Patent 4,816,571.

10 The methods of the invention can be used for the preparation of a variety of phosphorus-linked oligonucleotide species, such as phosphodiester, phosphorothioate, phosphorodithioate and H-phosphonate oligonucleotides.

15 The methods of the invention can be used to prepare several types of oligonucleotides, including phosphodiesters, phosphotriesters, phosphorothioates, phosphodithioates, and H-phosphonates. These oligonucleotides are of the structure:

20



wherein Z is an acid labile protecting group and B is a naturally occurring nucleobase (i.e., adenine (A), cytosine (C), guanine (G), thymine (T) or uracil (U)) or any of the nucleobase analogs known in the art as described below. For 25 the phosphodiester DNA, X = H, and B = A, C, G, or T; and

for the phosphodiester RNA, X = OH, and B = A, C, G or U. Y is H, OH, SH or alkyl, and L is O or S. T is a solid support as described below for solid phase synthesis or a base labile protecting group for solution phase synthesis.

5 In the context of the present invention, the term "phosphorus-linked oligomer" refers to a plurality of joined nucleobase-bearing sugar moieties connected by a linking group having a phosphorus atom. Linking groups include phosphodiester, phosphotriester, phosphorothioate, 10 phosphodithioate, and H-phosphonate linkages.

The structures listed above are representative of commonly synthesized phosphorus-linked oligomers, and the application of the methods of the present invention to them is illustrative, and not limiting. For example, it is known 15 to substitute a wide variety of modifications on the above structures including base modifications, backbone modifications, phosphate modifications, sugar modifications, and 2' modifications. Recent modifications include replacing the sugar with an alternative structure which has 20 primary and a secondary alcohol groups similar to those of ribose. As used herein, these modified compounds are included within the definition of the term "phosphorus-linked oligomers".

As used herein, the term "5'-protected activated phosphorus compound" is intended to denote a mono-, di- or polynucleoside species that has an activated phosphorus group at its 3'-terminus, and bears a 5'-hydroxyl protecting group. The activated phosphorus group is one that is known in the art to undergo a coupling reaction with the 30 deprotected 5'-hydroxyl of a growing oligomeric chain according to standard synthetic methodologies, such as, for example, the phosphoramidite, phosphotriester and H-phosphonate synthetic methods. See for example Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 35 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents Nos. 4,725,677 and Re. 34,069; Sekine, M., etc al., *J. Org. Chem.*, 1979, 44, 2325; Dahl, O., *Sulfur Reports*, 1991, 11,

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167-192; Kresse, J., et al. ., *Nucleic Acids Research*, 1975, 2, 1-9; Eckstein, F., *Ann. Rev. Biochem.*, 1985, 54, 367-402; and Yau, E.K. U.S. Patent No. 5,210,264, and
Oligonucleotides and Analogues A Practical Approach,
5 Eckstein, F. Ed., IRL Press, New York, 1991, each of the disclosures of which are hereby incorporated by reference in their entirety. Thus, activated phosphorus groups include groups of formula -P(-O-Pr)-N(i-pr)₂, where Pr is a phosphorus protecting group useful in phosphoramidite synthesis, such
10 10 β -cyanoethyl.

In accordance with the methods of the invention, a 5'-O-protected nucleoside synthon is first attached to a solid support. Solid supports are substrates which are capable of serving as the solid phase in solid phase
15 synthetic methodologies, such as those described in Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents Nos. 4,725,677 and Re. 34,069. Linkers are known in the art as short molecules which serve to connect a solid support to
20 functional groups (e.g., hydroxyl groups) of initial synthon molecules in solid phase synthetic techniques. One such linker is a succinamide linker. Other suitable linkers are disclosed in, for example, *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y., 1991,
25 Chapter 1, pages 1-23.

Solid supports according to the invention include those generally known in the art to be suitable for use in solid phase methodologies, including, for example, controlled pore glass (CPG), oxalyl-controlled pore glass
30 (see, e.g., Alul, et al., *Nucleic Acids Research* 1991, 19, 1527), TentaGel Support (an aminopolyethyleneglycol derivatized support (see, e.g., Wright, et al., *Tetrahedron Letters* 1993, 34, 3373)) and Poros (a copolymer of polystyrene/divinylbenzene).

35 In accordance with the methods of the invention, after the initial nucleoside synthon is attached to the solid support, the 5'-hydroxyl of the nucleoside deprotected

with a deprotecting reagent, which includes a protic acid in a solvent, and, optionally, an additive.

Heretofore, deblocking of 5'-hydroxyl groups has been accomplished using such protic acids in a halogenated alkyl solvent such as dichloromethane or dichloroethane. However the use of such halogenated alkyl solvents is greatly disadvantageous because they are not easily disposed of (and therefore expensive to use) because of the environmental hazard they pose. For example, methylene chloride has been classified as a carcinogen by OSHA, and such low boiling solvents require a relatively large investment in recycling equipment.

It has been discovered in accordance with the present invention that deblocking reactions can be performed in solvents other than halogenated alkyl solvents. Thus, in accordance with preferred embodiments of the invention, deprotection (i.e., deblocking) of the 5'-O- protecting group is accomplished using a protic acid such as formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid, in an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent.

In more preferred embodiments, the solvent is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether. In still more preferred embodiments the solvent is benzene, toluene or *o*-, *m*- or *p*-xylene, with toluene being particularly preferred.

In some preferred embodiments, the solvent is high boiling; i.e., it has a boiling point greater than about 60°C. Such solvents are additionally advantageous in that they do not require a substantial investment in recycling equipment that meets stringent environmental regulations required by OSHA for lower boiling solvents.

As used herein, the term alkyl includes but is not limited to straight chain, branch chain, and alicyclic

hydrocarbon groups. Alkyl groups of the present invention may be substituted. Representative alkyl substituents are disclosed in United States Patent No. 5,212,295, for example at column 12, lines 41-50, the disclosure of which is hereby 5 incorporated by reference in its entirety.

The term aromatic solvent is intended to denote aromatic compounds that are known to be useful as solvents in the art. These include for example, substituted and unsubstituted benzene, pyrrole, furan, and tetrahydrofuran, 10 where the substituents include halogen, nitro, or hydroxymethyl.

The term alkyl aromatic solvent is intended to denote aralkyl compounds (i.e., compounds that have one or more alkyl groups attached to an aromatic ring) that are 15 known to be useful as solvents in the art. These include for example, toluene, *o*-, *m*- or *p*-xylene, and mesitylene. Also included within the definition of "alkyl aromatic solvent" are liquid aromatic ring compounds that contain cyano groups, such as benzonitrile.

20 Halogens include fluorine, chlorine, bromine and iodine.

The term aromatic ether solvent is intended to mean a compound of formula R-O-R, wherein R is phenyl, halophenyl, or heteroaromatic, where the term heteroaromatic 25 denotes an aromatic compound having at least one heteroatom (i.e., an atom other than carbon) in the aromatic ring.

The deprotected 5'-hydroxyl of the support bound nucleoside, or growing oligomeric chain, is then reacted with a the 5'-protected activated phosphorus compound to 30 produce a covalent linkage therebetween. In some preferred embodiments wherein the activated phosphorus compound is a phosphoramidite, a phosphite linkage is produced. In other preferred embodiments, such as in H-phosphonate chemistries, the activated phosphorus group has the formula 35 -O-HP(=O)-O-, and reacts with a free 3'-hydroxyl of the growing oligonucleotide chain in the presence of an activating agent such as pivaloyl chloride to produce an

H-phosphonate linkage.

In some preferred embodiments, a sulfurization step or oxidation step is performed after each deprotection-coupling cycle. However, if desired, oxidation or 5 sulfurization can be performed in a single step at the end of the iterative synthesis.

Useful oxidizing agents according to the present invention include iodine, t-butyl hydroperoxide, or other oxidizing reagents known in the art.

10 Sulfurizing agents used during oxidation to form phosphorothioate linkages include Beaucage reagent (see e.g. Iyer, R.P., et al. *J. Chem. Soc.*, 1990, 112, 1253-1254, and Iyer, R.P., et al., *J. Org. Chem.*, 1990, 55, 4693-4699); tetraethylthiuram disulfide (see e.g., Vu, H., Hirschbein, 15 B.L., *Tetrahedron Lett.*, 1991, 32, 3005-3008); dibenzoyl tetrasulfide (see e.g., Rao, M.V., et al. *Tetrahedron Lett.*, 1992, 33, 4839-4842); di(phenylacetyl)disulfide (see e.g., Kamer, P.C.J., *Tetrahedron Lett.*, 1989, 30, 6757-6760); 1,2,4-dithiazoline-5-one (DtsNH) and 3-ethoxy-1,2,4-20 dithiazoline-5-one (EDITH) and (see Xu et al., *Nucleic Acids Research*, 1996, 24, 3643-3644 and Xu et al., *Nucleic Acids Research*, 1996, 24, 1602-1607); thiophosphorus compounds such as those disclosed in U.S. patent No. 5,292,875 to Stec et al., and U.S. patent No. 5,151,510 to 25 Stec et al., disulfides of sulfonic acids, such as those disclosed in Efimov et al., *Nucleic Acids Research*, 1995, 23, 4029-4033, sulfur, sulfur in combination with ligands like triaryl, trialkyl, triaralkyl, or trialkaryl phosphines.

30 The deprotection and coupling steps, and, optionally, oxidation or sulfurization steps, are repeated using mono-, di- or polymeric activated synthons until the desired base sequence is achieved. The completed oligomer is then cleaved from the solid support. Cleavage is 35 achieved by any of the standard methods in the art, such as, for example, with concentrated ammonium hydroxide.

The methods of the present invention can be used

for the synthesis of phosphorus-linked oligonucleotides having both naturally occurring and non-naturally occurring constituent groups. For example, the present invention can be used to synthesize phosphodiester, phosphorothioate, 5 phosphorodithioate, and H-phosphonate oligomers having naturally occurring pentose sugar components such as ribose and deoxyribose, and their substituted derivatives, as well as other sugars known to substitute therefor in oligonucleotide analogs.

10 The constituent sugars and nucleosidic bases of the phosphorus-linked oligonucleotides can be naturally occurring or non-naturally occurring. Non-naturally occurring sugars and nucleosidic bases are typically structurally distinguishable from, yet functionally inter-
15 changeable with, naturally occurring sugars (e.g. ribose and deoxyribose) and nucleosidic bases (e.g., adenine, guanine, cytosine, thymine). Thus, non-naturally occurring nucleobases and sugars include all such structures which mimic the structure and/or function of naturally occurring species, and which aid in the binding of the oligonucleotide to a target, or which otherwise advantageously contribute to the properties of the oligonucleotide.
20

The methods of the invention are amenable to the synthesis of phosphorus-linked oligomers having a variety of substituents attached to their 2'-positions. These include, for example, halogens, O-alkyl, O-alkylamino, O-alkylalkoxy, protected O-alkylamino, O-alkylaminoalkyl, O-alkyl imidazole, and polyethers of the formula (O-alkyl)_m, where m is 1 to about 10. Preferred among these polyethers are linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and those which are disclosed by Ouchi, et al., *Drug Design and Discovery* 1992, 9, 93, Ravasio, et al., *J. Org. Chem.* 1991, 56, 4329, and Delgado et. al., *Critical Reviews in Therapeutic Drug Carrier Systems* 1992, 9, 249. Further sugar modifications are disclosed in Cook, P.D., *supra*. Fluoro, O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl

amino substitution is described in United States Patent Application serial number 08/398,901, filed March 6, 1995, entitled Oligomeric Compounds having Pyrimidine Nucleotide(s) with 2' and 5' Substitutions, the disclosure 5 of which is hereby incorporated by reference. Further representative O-substitutions on the ribosyl ring include S, CH₂, CHF, and CF₂, see, e.g., Secrist, et al., *Abstract 21, Program & Abstracts, Tenth International Roundtable, Nucleosides, Nucleotides and their Biological Applications*, 10 Park City, Utah, Sept. 16-20, 1992.

Representative nucleobases suitable for use in the methods of the invention include adenine, guanine, cytosine, uridine, and thymine, as well as other non-naturally occurring and natural nucleobases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halo uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo, oxa, amino, thiol, thioalkyl, hydroxyl 20 and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine. Further naturally and non naturally occurring nucleobases include those disclosed in U.S. Patent No. 3,687,808 (Merigan, et al.), in chapter 15 by Sanghvi, 25 in *Antisense Research and Application*, Ed. S. T. Crooke and B. Lebleu, CRC Press, 1993, in Englisch et al., *Angewandte Chemie, International Edition*, 1991, 30, 613-722 (see especially pages 622 and 623, and in the *Concise Encyclopedia of Polymer Science and Engineering*, J.I. 30 Kroschwitz Ed., John Wiley & Sons, 1990, pages 858-859, Cook, P.D., *Anti-Cancer Drug Design*, 1991, 6, 585-607. The terms "nucleosidic base" and "nucleobase" are further intended to include heterocyclic compounds that can serve as nucleosidic bases, including certain 'universal bases' that 35 are not nucleosidic bases in the most classical sense, but function similarly to nucleosidic bases. One representative example of such a universal base is 3-nitropyrrole.

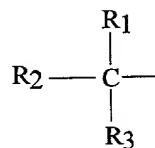
The methods of the present invention use labile protecting groups to protect various functional moieties during synthesis. Protecting groups are used ubiquitously in standard oligonucleotide synthetic regimes for protection 5 of several different types of functionality. In general, protecting groups render chemical functionality inert to specific reaction conditions and can be appended to and removed from such functionality in a molecule without substantially damaging the remainder of the molecule. See, 10 e.g., Green and Wuts, *Protective Groups in Organic Synthesis*, 2d edition, John Wiley & Sons, New York, 1991. Representative protecting groups useful to protect nucleotides during synthesis include base labile protecting groups and acid labile protecting groups. Base labile 15 protecting groups are used to protect the exocyclic amino groups of the heterocyclic nucleobases. This type of protection is generally achieved by acylation. Two commonly used acylating groups for this purpose are benzoylchloride and isobutyrylchloride. These protecting groups are stable 20 to the reaction conditions used during oligonucleotide synthesis and are cleaved at approximately equal rates during the base treatment at the end of synthesis.

In some preferred embodiments, the 5'-protected activated phosphorus compound is a nucleoside 25 phosphoramidite. Phosphoramidites of numerous nucleosides and derivatized solid supports are commercially available through various companies (e.g. Applied Biosystems Inc., Millipore Corp.). The amino moiety of such phosphordiamidites can be selected from various amines 30 presently used for phosphoramidites in standard oligonucleotide synthesis. These include both aliphatic and heteroalkyl amines. One preferred amino group is diisopropylamino. Other examples of suitable amines as are described in various United States patents, principally 35 those to M. Caruthers and associates. These include United States patents 4,668,777; 4,458,066; 4,415,732; and 4,500,707; all of which are herein incorporated by

reference.

Hydroxyl protecting groups typically used in oligonucleotide synthesis may be represented by the following structure:

5



wherein each of R_1 , R_2 and R_3 is an unsubstituted or mono-substituted aryl or heteroaryl group selected from phenyl, naphthyl, anthracyl, and five or six membered heterocyclic rings with a single heteroatom selected from N, O and S, or

10 two N heteroatoms, including quinolyl, furyl, and thienyl; where the substituent is selected from halo (i.e., F, Cl, Br, and I), nitro, $\text{C}_1\text{-C}_4$ -alkyl or alkoxy, and aryl, aralkyl and cycloalkyl containing up to 10 carbon atoms; and wherein R_2 and R_3 may each also be $\text{C}_1\text{-C}_4$ -alkyl or aralkyl or
 15 cycloalkyl containing up to 10 carbon atoms.

In preferred embodiments of the invention, the 5'-protecting group is trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotriptyl, DATE, TBTr, Pixyl or Moxyl, with trityl, monomethoxy trityl, 20 dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl MOX) being more preferred, and with dimethoxy trityl being especially preferred.

In some preferred embodiments, the present invention provides methods for the preparation of a
 25 phosphorus-linked oligomer comprising the steps of:

- a) providing a solid support;
- b) attaching a 5'-O-protected nucleoside to the solid support;
- c) contacting the protected 5'-hydroxyl of the
 30 nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl

aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;

d) reacting the deprotected 5'-hydroxyl with a 5' -protected activated phosphite compound to produce a phosphite linkage;

e) oxidizing or sulfurizing the phosphite linkage to form a phosphodiester, phosphorothioate, or phosphorodithioate linkage;

10 f) repeating steps c through e at least once for subsequent couplings of additional activated phosphite compounds, to produce the completed phosphorus-linked oligomer; and

g) cleaving the oligomer from the solid support.

15 As used herein, the term "activated phosphite compound" is intended to include mono-, di- and polynucleoside phosphoramidites.

As used herein, the term "5'-protected activated phosphate compound" is intended to include a 5'-protected mono-, di- and polynucleoside phosphoramidite, as is used in standard solid phase oligonucleotide synthesis.

In some preferred embodiments of the invention the phosphordiamidite is activated to nucleophilic attack by the 5' hydroxyl by use of an activating agent. It is believed 25 that the activating agent displaces one of the amino groups from the phosphordiamidite, thereby rendering the phosphorus of the phosphordiamidite more susceptible to nucleophilic attack by the 5' hydroxyl group of the growing nucleotide chain. Any activating agent that can activate the 30 phosphorous to nucleophilic attack without interacting with the growing nucleotide chain may be suitable for use with the present invention. One preferred activating agent is tetrazole. Some commonly used commercially available activating agents are thiotetrazole, nitrotetrazole, and 35 N,N-diisopropylaminohydrotetrazolide. Other suitable activating agents are also disclosed in the above incorporated patents as well as in United States patent

4,725,677 and in Berner, S., Muhlegger, K., and Seliger, H., *Nucleic Acids Research* 1989, 17:853; Dahl, B.H., Nielsen, J. and Dahl, O., *Nucleic Acids Research* 1987, 15:1729; and Nielson, J. Marugg, J.E., Van Boom, J.H., Honnens, J., 5 Taagaard, M. and Dahl, O., *J. Chem. Research* 1986, 26, all of which are herein incorporated by reference.

Phosphorus linked oligomers produced by the methods of the invention will preferably be hybridizable to a specific target oligonucleotide. Preferably, the phosphorus 10 linked oligomers produced by the methods of the invention comprise from about 1 to about 100 monomer subunits. It is more preferred that such compounds comprise from about 10 to about 30 monomer subunits, with 15 to 25 monomer subunits being particularly preferred.

15 As will be recognized, the process steps of the present invention need not be performed any particular number of times or in any particular sequence. Additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon 20 examination of the following examples thereof, which are not intended to be limiting.

Example 1

Synthesis of 5'-TTTTTTT-3' phosphorothioate heptamer

50 milligram (2 μ mole) of 5'-O-
25 dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage is taken up in a glass reactor, and a toluene solution of 3% dichloroacetic acid (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M
30 solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a
35 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-

picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile, and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

This complete cycle is repeated five more times to produce the completely protected thymidine heptamer. The carrier containing the compound is treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature. The aqueous solution is filtered, and concentrated under reduced pressure to give a phosphorothioate heptamer, TTTTTTT.

15 **Example 2**

Synthesis of 5'-d(GACT)-3' phosphorothioate tetramer

50 milligram (2 μ mole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage is taken up in a glass reactor, and a toluene solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF

is added to cap the unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group.

5 The product is washed with acetonitrile. Then, a 0.2 M solution of N^4 -benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and 10 allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is 15 repeated one more time for 3 minutes. The support is washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

20 A solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M solution of N^6 -benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in anhydrous acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and 25 allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room 30 temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF 35 is added to cap the unreacted 5'-hydroxyl group. The product

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is washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M 5 solution of N^2 -isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and allowed to react at room temperature for 5 minutes. The 10 product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed 15 with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

The carrier containing the compound is treated with 20 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hour. The aqueous solution is filtered, concentrated under reduced pressure to give a phosphorothioate tetramer of 5'-dG-dA-dC-T-3'.

25 **Example 3**

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale 30 using the cyanoethyl phosphoramidites and Pharmacia's primary support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v)

for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

5 **Example 4**

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale 10 using the cyanoethyl phosphoramidites and Pharmacia's primary support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the 15 end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

Example 5

20 **Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21-mer**

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale 25 using the cyanoethyl phosphoramidites and Pharmacia's primary support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the 30 end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

Example 6**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

The synthesis of the above sequence was performed on a 5 Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide 10 in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 $^{\circ}$ C for 24 hours to give the product.

15 **Example 7**

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2' -methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Milligen 8800 Synthesizer on a 282 μ mole scale using 20 cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end 25 of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 $^{\circ}$ C for 24 hours to give the product.

Example 8**30 Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2' -methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer**

The synthesis of the above sequence was performed on a

Pharmacia OligoPilot II Synthesizer on a 250 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primary support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was 5 performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 10 hours to give the product.

It is intended that each of the patents, applications, printed publications, and other published documents mentioned or referred to in this specification be herein incorporated by reference in their entirety.

15 Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is therefore intended that the 20 appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method for the preparation of a phosphorus-linked oligomer comprising the steps of:
 - (a) providing a solid support;
 - 5 (b) attaching a 5'-O-protected nucleoside to the solid support;
 - (c) deprotecting the 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the 10 solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;
 - (d) reacting the deprotected 5'-hydroxyl with an 5'-protected activated phosphorus compound to produce a 15 covalent linkage therebetween;
 - (e) oxidizing or sulfurizing the covalent linkage to form a phosphodiester, phosphorothioate, phosphorodithioate or H-phosphonate linkage;
 - (f) repeating steps c through e at least once for 20 subsequent couplings of additional activated phosphorus compounds, to produce the completed phosphorus-linked oligomer; and
 - (g) cleaving the oligomer from the solid support.
2. The method of claim 1 further comprising the step 25 of capping remaining reactive sites with a solution containing a capping reagent.
3. The method of claim 1 wherein the oxidation or sulfurization step is performed after each iteration of steps (c) and (d).
- 30 4. The method of claim 1 wherein the oxidation or sulfurization step is performed after the final iteration of steps (c) and (d).
5. The method of claim 1 wherein the solvent in step

(c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether.

6. The method of claim 1 wherein the solvent in step
(c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene,
5 mesitylene, or diphenyl ether.

7. The method of claim 6 wherein the solvent in step (c) is benzene, toluene or *o*-, *m*- or *p*-xylene.

8. The method of claim 7 wherein the solvent in step (c) is toluene.

10 9. The method of claim 1 wherein the solvent in step
(c) is a halogenated aromatic solvent or a halogenated alkyl
aromatic solvent.

10. The method of claim 9 wherein the solvent in step (c) is chlorobenzene or benzotetrafluoride.

15 11. The method of claim 1 wherein the activated phosphorus compound is an activated mononucleotide, an activated dinucleotide, or an activated polynucleotide.

12. The method of claim 1 wherein the activated phosphorus compound is a 5'-protected nucleoside phosphoramidite or a 5'-protected activated H-phosphonate nucleoside.

13. The method of claim 1 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl,
25 monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotriptyl, DATE, TBTr, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX).

14. The method of claim 13 wherein the protecting

group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl.

5 15. The method of claim 14 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl.

16. The method of claim 1 wherein the phosphorus-linked oligomer is a phosphodiester, phosphorothioate
10 phosphorodithioate, or H-phosphonate oligonucleotide.

17. The method of claim 1 wherein the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or
15 phenylphosphoric acid.

18. The method of claim 1 wherein the solvent in step (c) further comprises an additive.

19. The method of claim 18 wherein the additive to the solvent in step (c) is an alcohol.

20 20. The method of claim 19 wherein the alcohol additive to the solvent in step (c) is from 0% to about 30% methanol, ethanol, 2-propanol, t-butyl alcohol, t-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof.

25 21. A method for the preparation of a phosphorus-linked oligomer comprising the steps of:

- a) providing a solid support;
- b) attaching a 5'-O-protected nucleoside to the solid support;
- 30 c) contacting the protected 5'-hydroxyl of the

nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a

5 halogenated alkyl aromatic solvent, or an aromatic ether solvent;

d) reacting the deprotected 5'-hydroxyl with a 5'-protected activated phosphite compound to produce a phosphite linkage;

10 e) oxidizing or sulfurizing the phosphite linkage to
form a phosphodiester, phosphorothioate, or
phosphorodithioate linkage;

f) repeating steps c through e at least once for subsequent couplings of additional activated phosphite compounds, to produce the completed phosphorus-linked oligomer; and

g) cleaving the oligomer from the solid support.

22. The method of claim 21 further comprising the step
of capping remaining reactive sites with a solution
20 containing a capping reagent.

23. The method of claim 21 wherein the solvent in step (c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether.

24. The method of claim 23 wherein the solvent in step
25 (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene,
mesitylene, or diphenyl ether.

25. The method of claim 24 wherein the solvent in step (c) is benzene, toluene or *o*-, *m*- or *p*-xylene.

26. The method of claim 25 wherein the solvent in step
30 (c) is toluene.

27. The method of claim 21 wherein the solvent in step

(c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent.

28. The method of claim 27 wherein the solvent in step (c) is chlorobenzene or benzotirfluoride.

5 29. The method of claim 21 wherein the activated phosphite compound is a mononucleotide phosphoramidite, a dinucleotide phosphoramidite, or a polynucleotide phosphoramidite.

10 30. The method of claim 21 wherein the protetcing group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotriyl, DATE, TBTr, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX).

15 31. The method of claim 30 wherein the protetcing group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl.

20 32. The method of claim 31 wherein the protetcing group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl.

25 33. The method of claim 21 wherein the phosphorus-linked oligomer is a phosphodiester, phosphorothioate or a phosphorodithioate oligonucleotide.

30 34. The method of claim 21 wherein the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid.

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35. The method of claim 21 wherein the solvent in step (c) further comprises an additive.

36. The method of claim 35 wherein the additive to the solvent in step (c) is an alcohol.

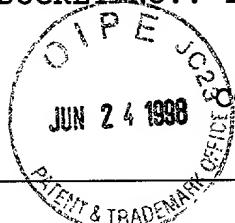
5 37. The method of claim 36 wherein the alcohol additive to the solvent in step (c) is from 0% to about 30% methanol, ethanol, 2-propanol, *t*-butyl alcohol, *t*-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof.

10 38. The method of claim 22 wherein the solvent in step (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether; the activated phosphite compound is a mononucleotide phosphoramidite, a dinucleotide phosphoramidite, or a polynucleotide phosphoramidite; the 15 protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl; the phosphorus linked oligomer is a phosphodiester, phosphorothioate or a phosphorodithioate oligonucleotide; and the protic acid is dichloroacetic acid.

20 39. The method of claim 38 wherein the solvent in step (c) is toluene.

40. The method of claim 39 wherein the activated phosphite compound is a mononucleotide phosphoramidite.

25 41. The method of claim 1 wherein the 5'-protected activated phosphorus compound is a 5'-protected activated H-phosphonate compound; and the phosphorus-linked oligomer is a H-phosphonate oligonucleotide.



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES** the specification of which:

() is attached hereto.

(X) was filed on February 26, 1998 as Application Serial No. 09/032,972 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **John W. Caldwell** and **Michael P Straher**, Registration Nos. 28,937 and 38,325 of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and **Herb Boswell, Laurel Bernstein and Andrew E. Granston**, Registration Nos. 27,311, 37,280 and 38,473, of **ISIS Pharmaceuticals**, 2292 Faraday Avenue, Carlsbad, California 92008.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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	Residence:	Citizenship:	
	Post Office Address:		

DOCKET NO. ISIS-2710

PATENT



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Achim H. Krotz and Vasulinga T. Ravikumar

Serial No.: 09/032,972

Group Art Unit: 1801

Filed: January 26, 1998

Examiner: Not Yet Assigned

For: IMPROVED PROCESS FOR
SYNTHESIS OF OLIGONUCLEOTIDES

Assistant Commissioner for Patents
Washington DC 20231

Sir:

ASSOCIATE POWER OF ATTORNEY

The undersigned, of the firm WOODCOCK WASHBURN KURTZ
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of **ISIS PHARMACEUTICALS, INC.**, 2292 Faraday Avenue, Carlsbad, California 92008; his/her associates with full power to prosecute the above-identified application and to transact all business in the Patent Office connected therewith and requests that correspondence continue to be directed to the firm of WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP at the above address.

Date: June 22, 1998



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Applicant or Patentee: Achim H. Krotz and Vasulinga T. Ravikumar

Serial No.: To Be Assigned Attorney's Docket No.: ISIS-2710

Date Filed: Herewith

For: IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN**

I hereby declare that I am:

the owner of the small business concern identified below:

an official empowered to act on behalf of the concern identified below:

NAME OF CONCERN: ISIS Pharmaceuticals, Inc.

ADDRESS OF CONCERN: 2292 Faraday Avenue
Carlsbad, CA 92008

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that: (1) the number of employees of the concern, including those of its affiliates, does not exceed 500 persons; and (2) the concern has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES** by inventor(s) **Achim H. Krotz and Vasulinga T. Ravikumar** described in

specification filed herewith.

application serial no. _____, filed _____

patent no. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

***NOTE: Separate verified statements are required for each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

FULL NAME:

ADDRESS:

INDIVIDUAL SMALL BUSINESS CONCERN NONPROFIT ORGANIZATION

FULL NAME:

ADDRESS:

INDIVIDUAL SMALL BUSINESS CONCERN NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING

Daniel L. Kisner

TITLE OF PERSON SIGNING

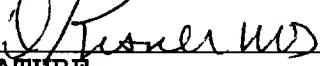
President & Chief Operating Officer

ADDRESS OF PERSON SIGNING

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Carlsbad, CA 92008

SIGNATURE



2/25/98

DATE